



Depletion of TDP-43 affects *Drosophila* motoneurons terminal synapsis and locomotive behavior

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ARTICLE INFO

Article history:

Received 30 January 2009

Revised 3 April 2009

Accepted 13 April 2009

Available online 19 April 2009

Edited by Jesus Avila

Keywords:

Drosophila

TDP-43

Als

Motoneurons

Synapsis

Locomotion

ABSTRACT

Pathological modifications in the highly conserved and ubiquitously expressed heterogeneous ribonucleoprotein TDP-43 were recently associated to neurodegenerative diseases including amyotrophic lateral sclerosis (ALS), a late-onset disorder that affects predominantly motoneurons [Neumann, M. et al. (2006) Ubiquitinated TDP-43 in frontotemporal lobar degeneration and amyotrophic lateral sclerosis. *Science* 314, 130–133, Sreedharan, J. et al. (2008) TDP-43 mutations in familial and sporadic amyotrophic lateral sclerosis. *Science* 319, 1668–1672, Kabashi, E. et al. (2008) TARDBP mutations in individuals with sporadic and familial amyotrophic lateral sclerosis. *Nat. Genet.* 40, 572–574]. However, the function of TDP-43 in vivo is unknown and a possible direct role in neurodegeneration remains speculative. Here, we report that flies lacking *Drosophila* TDP-43 appeared externally normal but presented deficient locomotive behaviors, reduced life span and anatomical defects at the neuromuscular junctions. These phenotypes were rescued by expression of the human protein in a restricted group of neurons including motoneurons. Our results demonstrate the role of this protein in vivo and suggest an alternative explanation to ALS pathogenesis that may be more due to the lack of TDP 43 function than to the toxicity of the aggregates.

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1. Introduction

TDP-43 is a highly conserved and ubiquitously expressed nuclear protein containing RNA binding motives and reported to be involved in pre-mRNA splicing, transcription, mRNA stability, and mRNA transport [4]. Recently, TDP-43 was identified as the main protein component of the intracellular inclusions observed in affected brain areas of patients suffering from Amyotrophic Lateral Sclerosis (ALS), Frontotemporal Lobar Degeneration with ubiquitinated inclusions (FTLD-U) [1,5,6] and Alzheimer disease (AD) [7–9]. In the brain of these individuals TDP-43 appears depleted from the cell nucleus and abnormally localized inside insoluble protein aggregates throughout the cytoplasm. Moreover, missense mutations in the C-terminal part of TDP-43 were identified in 2–5% of sporadic and familial forms of ALS indicating that a tight correlation between TDP-43 protein function and neurodegenerative diseases may exist [2,3].

Despite the fact that the biochemical and structural properties of TDP-43 were extensively studied, its physiological role in vivo or the possible pathological mechanisms that may lead to neuronal diseases were not determined [10]. Regarding this, considerable attention has been given to the potential toxic effect of the TDP-

43 protein aggregates observed in the cytoplasm. However it should also be considered that in those neurons TDP-43 seems to be absent from the nucleus. Therefore, it might be possible that the defects observed in ALS patients reflect a loss of TDP-43 nuclear function rather than a pure toxic effect of its aggregates.

We have previously shown that the *Drosophila* TDP-43 homolog was able to replace the splicing function of the human protein in vitro and in tissue culture cells [11,12], for that reason we decided to investigate the role of TDP-43 in vivo using *Drosophila melanogaster* as the animal model.

2. Material and methods

2.1. Generation of *TBPH* null alleles

TBPH loss-of-function alleles were generated by imprecise mobilization of TBPH^{EY10530} transposon using Δ2–3 transposase. Potential candidates were identified by the loss of w⁺ markers and balanced to stock. Genomic DNA from 450 lines was used as polymerase chain reaction templates. Primers flanking the EY10530 insertion were used for mapping (1R:19750253–19750232; 2R:19750667–19750640; 1F:19746555–19746576; 3F:19747048–19747071; 4F:19748563–19748583; 5F:19749069–19749089; 6F:19749727–19749746). TBPH^{Δ23} revealed a deletion of 1616 bp with complete elimination of the P-element and break

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points at 19748477–19750093. In the case of $TBPH^{\Delta 142}$, the deletion is from 19749289 to 19750093 including a fragment of EY10530 imprecisely excised (1138 bp).

2.2. Preparation of TBPH antibody

TBPH protein fragment from amino acids 1–268 GST conjugated was expressed and purified from *Escherichia coli* over a GST-resin following supplier protocols (Clontech #635610) and utilized to immunize rabbits according to standard immunization protocols.

2.3. Western blot analysis

Drosophila heads were squeezed in lysis buffer (10 mM Tris-HCl pH 7.4, 150 mM NaCl, 5 mM EDTA, 5 mM EGTA, 10% Glycerol, 50 mM NaF, 5 mM DTT, 4 M Urea, and protease inhibitors (Roche Diagnostic #11836170001). The proteins separated by 8% SDS-PAGE, were transferred to nitrocellulose membranes (GE Healthcare #RPN303E) and probed with primary antibodies: TBPH (1:3000) and tubulin (1:2000; Calbiochem #CP06). Secondary antibodies: (HRP)-labeled anti-mouse or anti-rabbit antibodies

(1:10000) (Pierce #31460; #31430)) and developed using ECL (#RPN2109; GE healthcare).

2.4. Immunohistochemistry

Larval body wall muscles were dissected in saline containing 0.1 mM Ca^{+2} and fixed in ice-cold 4% paraformaldehyde for 20 min, washed in PBS/0.1% Tween 20, blocked with 5% Normal Goat Serum (NGS Chemicon #S26-100 ML) 30 min. Primary Anti-HRP antibody (Jackson Immunoresearch laboratories, #323-005-021) (1:100) over night at 4 °C. Secondary anti-rabbit antibody conjugated with Alexa 488 (1:500) (Invitrogen #411008) or Phalloidin TRITC (1:500 dilution) for 2 h.

2.5. NMJ analysis

Wandering third instar larvae were processed as before and the number of terminals branches and type 1b (big) and 1s (small) boutons present in muscle 6/7 from abdominal segments 2 to 4 were quantified. To obtain the images from the synaptic terminals, a 12–15 μ m deep Z-series containing the entire NMJ were collected with a confocal laser-scanning microscope.

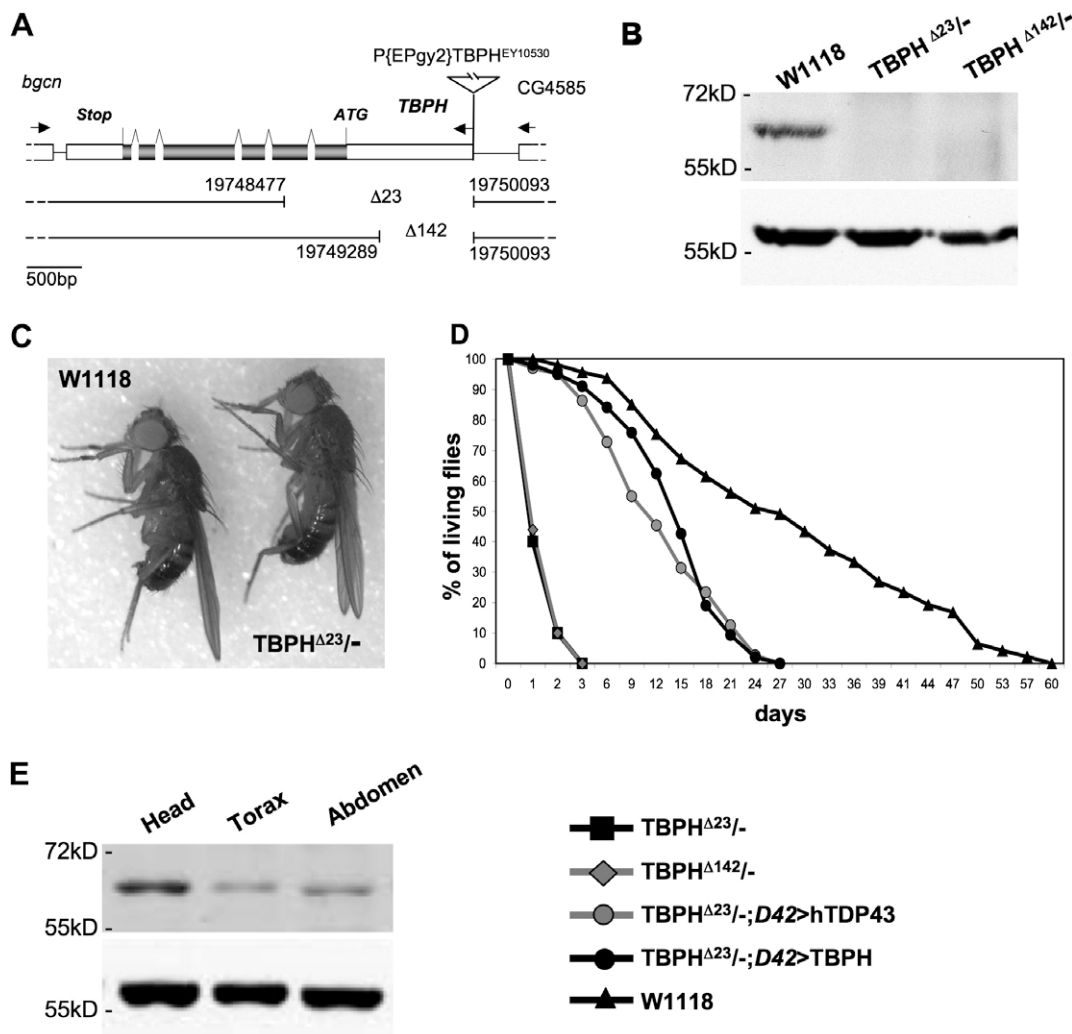


Fig. 1. Depletion of *Drosophila* TBPH affects locomotive behavior and life span. (A) Schematics of TBPH mutant alleles. (B) Western blot stained with anti-TBPH antibody, detects no endogenous protein in mutant fly heads (upper panel) tubulin was a loading control (bottom panel). (C) Wild type controls and TBPH mutant flies appear externally identical. (D) Life span reduction in TBPH mutant flies can be rescued by expression of either *drosophila* or human TDP-43 transgene in *D42*-GAL4 expressing neurons. (E) Western blot analysis shows higher TBPH protein levels in fly heads compared to torax or abdomen (upper panel). Tubulin was a loading control (bottom panel).

2.6. Eclosion analysis

Embryos from $TBPH^{\Delta 23}/CyO^{GFP}$, $TBPH^{\Delta 142}/CyO^{GFP}$ and Oregon R flies were collected during two hours at 25°. Homo or heterozygous larvae, identified based on the absence or presence of GFP expression, were selected between 26 and 28 h after egg laying using a fluorescent stereo microscope. Hatched first instar wild type, $TBPH$ homozygous and heterozygous larvae were collected, placed into new vials in batches of 25–30 larvae per vial, and grown at 25°C. Developmental viability was calculated as percentage of hatched first instar larvae that survived to pupae and adulthood.

2.7. Transgenic flies and genetic rescue experiments

Endogenous $TBPH$ and Human TDP-43 cDNAs were Flag tagged and cloned in modified 10×UAS vector (*EcoRI-XbaI*) to generate transgenic flies by standard embryo injections (Best Gene Inc.). Insertion lines on the 3rd chromosome were combined with 2nd chromosome $TBPH^{\Delta 23}$ allele using second and third chromosome compound balancer (ST). $TBPH^{\Delta 23};UASHTDP-43/ST$ females were

crossed against $TBPH^{\Delta 23};D42-GAL4/ST$ or $TBPH^{\Delta 23};elav-GAL4/ST$ males and the number of $TBPH^{\Delta 23}$ homozygous flies in the progeny analyzed.

2.8. RNAi treatment in vivo and fly stocks

The double strand RNAi against $TBPH$ were obtained from VDRC Vienna (ID38377 and ID38379) and target the genomic sequence situated in the chromosomal position 19748365–19748665 that corresponds to the $TBPH$ amino acid sequences 81–180. These lines were crossed against *elav-GAL4* and *1407-GAL4* in wild type or $TBPH^{\Delta 23}$ heterozygous backgrounds. All crosses were done at 29°C. All the stocks utilized here (*elav-GAL4*, *1407-GAL4*, *D42-GAL4*, $\Delta 2-3-Sb/TM3$ transposase and *UAScd8GFP*), were obtained from Bloomington Drosophila Stock Center.

2.9. Larval movement

One hundred and twenty hours aged larvae were selected individually, washed briefly with distilled water to remove any remaining food and carefully transferred to a LB agar plate under

Table 1
Developmental viability analysis of $TBPH$ mutant flies.

Genotypes	1st Instar larvae (28 h) N	Pupa		Eclosed flies		Trapped flies		Motility defects (%)
TBPH ^{Δ23} /CyO ^{GFP}	1127	734	65%	586	52%	26	2%	0
TBPH ^{Δ142} /CyO ^{GFP}	1056	616	58%	446	42%	42	4%	0
TBPH ^{Δ23} /TBPH ^{Δ23}	930	591	64%	197	21%	295	32%	100
TBPH ^{Δ142} /TBPH ^{Δ142}	539	327	61%	86	16%	172	32%	100
OregonR	758	628	83%	543	72%	15	2%	0

Quantitative analysis of $TBPH$ minus flies from embryonic stages to adulthood indicates that this mutation induce little larval or pupal, -but not embryonic-, lethality. Nevertheless, homozygous flies present serious motility defects during eclosion and adulthood.

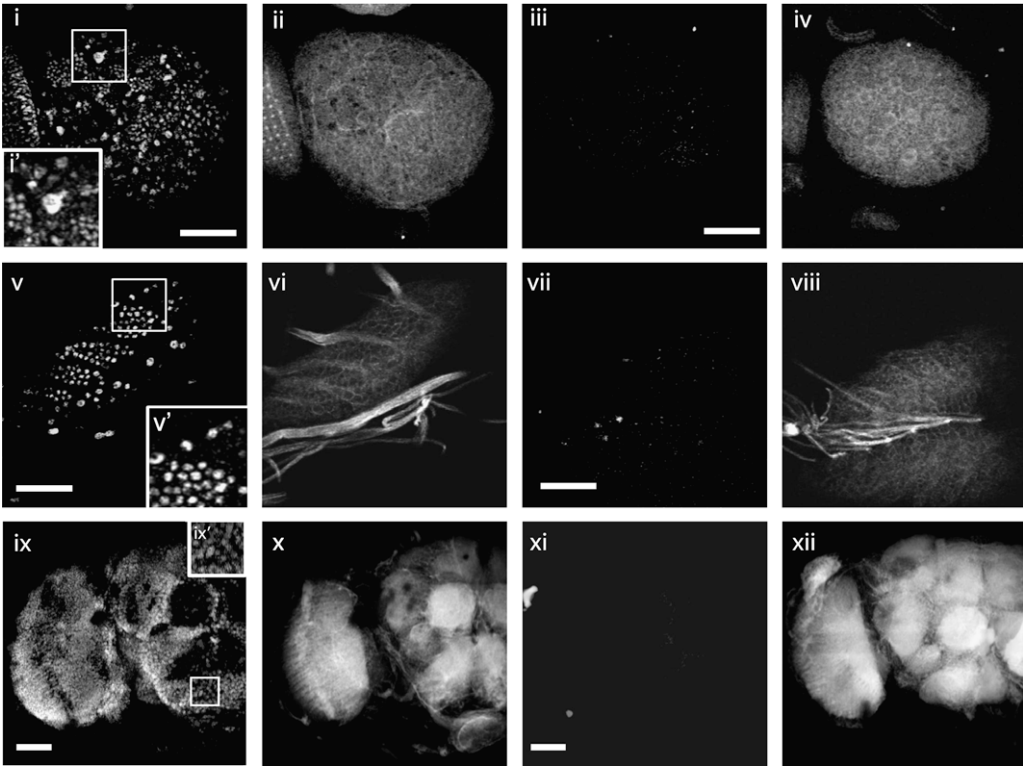


Fig. 2. Endogenous $TBPH$ protein is present in larval and adult brains. Anti- $TBPH$ immunostaining in wild type larval (i, v) and adult (ix) brains show the intracellular localization of the endogenous protein. Squares (i', v', ix') are higher magnifications. Signal disappear in $TBPH^{\Delta 23}$ homozygous brains (iii, vii and xi). ii, vi, x, iv, viii and xii are phalloidin staining. Scale: 50 μ m.

stereoscope. Larvae were allowed to adapt for 30 s and the number of peristaltic waves during the period of two minutes time were counted. Around 30 larvae were counted for each genotype and separate LB agar plate was used for each genotype.

2.10. Climbing assay

Newly eclosed males were transferred in batches of 30 to fresh vials and aged for 3–4 days. They were transferred, without anesthesia, to a 15 ml conical tube, tapped to the bottom of the tube, and their subsequent climbing activity quantified as the percentage of flies that reach the top of the tube in 15 s.

2.11. Walking assay

Newly eclosed males were transferred in batches of 15 to fresh vials and aged for 3–4 days. Individual flies were placed in the center of 145 mm Petri dish marked with 1 cm square grid. Locomotion was quantified as the number of grid line crossings during 30 s.

2.12. Life span

Adult flies were collected during two days and transferred to fresh tubes at a density of 20 per vial with a proportion of 10 male

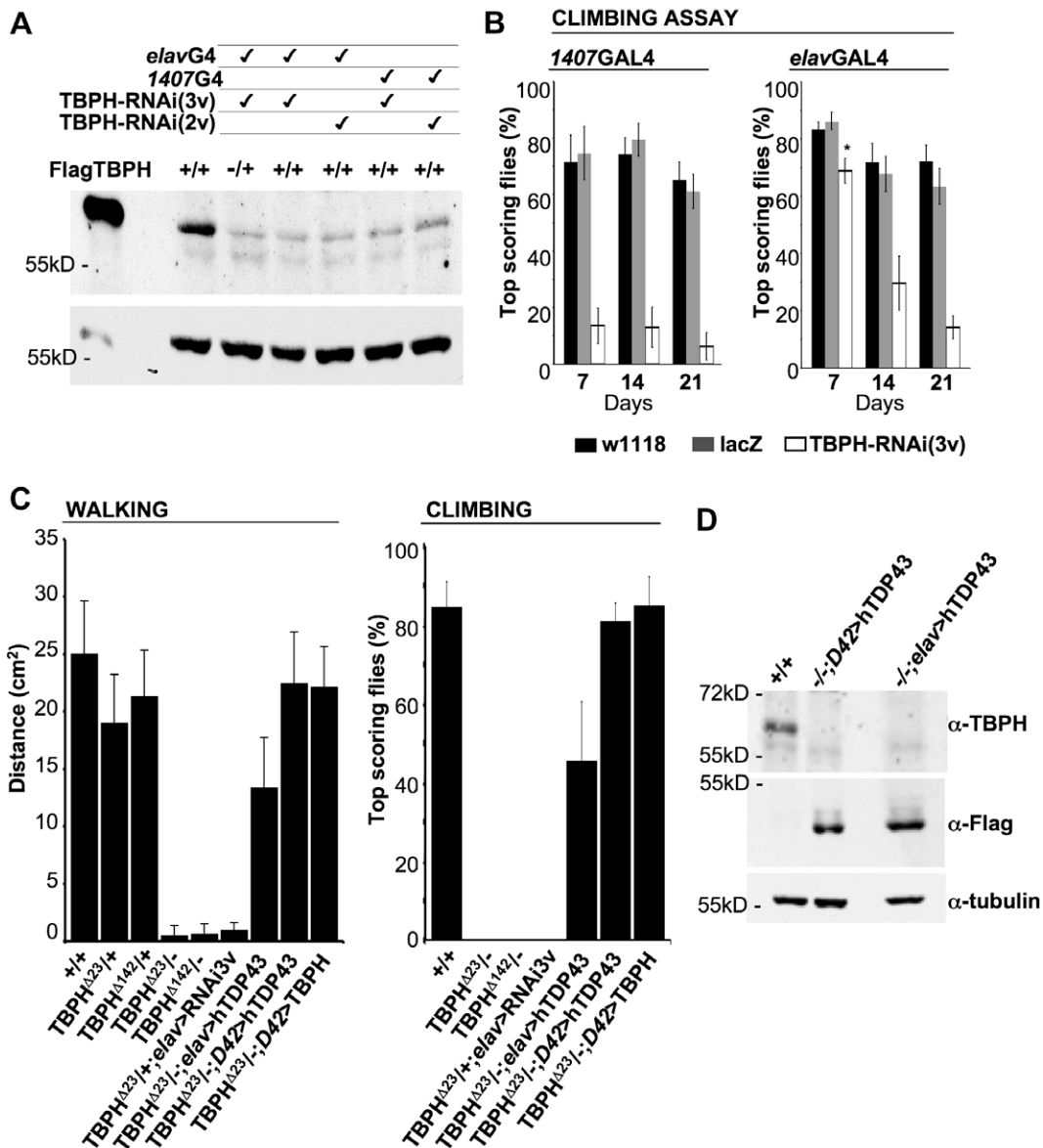


Fig. 3. Presynaptic TBPH function regulates flies locomotion. (A) Expression of two different TBPH RNAi insertions in neurons using 1407-GAL4 and elav-GAL4 reduced endogenous TBPH protein in adult heads (upper blot). First line shows TBPH transfected S2 cell extracts as a positive control. (+/+) states for wild type backgrounds while (-/-) is for TBPH^{Δ23} heterozygous flies. Tubulin was internal loading control (lower blot). (B) Climbing defects in TBPH RNAi treated flies during time, 1407-GAL4 > TBPH-RNAi (left) and elav-GAL4 > TBPH-RNAi (right), compared to wild type or GAL4 flies expressing UAS-LacZ. *n* = 100 flies for genotype, error bars indicate S.D.; asterisk *P* < 0.0001 by ANOVA single factor. (C) TBPH homozygous or heterozygous alleles treated with elav-GAL4 > TBPH RNAi (forth, fifth and sixth lanes of the left graph), present impaired spontaneous walking and climbing activities (second, third and fourth lanes of the right graph). These defects become suppressed by expressing hTDP-43 with the pan-neuronal elav-GAL4 or the more restricted neuronal driver D42-GAL4. Similar rescue was obtained with the endogenous TBPH protein (last three lanes). *n* = 50 flies for each genotype, error bars indicate S.D.; *P* < 0.0001 calculated by ANOVA single factor. (D) Western blot analysis of rescued flies. TBPH^{Δ23}/TBPH^{Δ23};D42-GAL4/UAShTDP-43 and TBPH^{Δ23}/TBPH^{Δ23};elav-GAL4/UAShTDP-43 flies were blotted using antibodies against TBPH (upper blot) and against Flag to label flagged hTDP-43 (middle blot). Observe that Flagged hTDP-43 strains do not express endogenous TBPH. Tubulin was the loading control (lower blot).

and 10 female. All the experiments were conducted in a humidified, temperature controlled incubator at 25 °C and 60% humidity on a 12-h light and 12 h dark cycle. Flies were fed with standard cornmeal (2.9%), sugar (4.2%), yeast (6.3%) fly food. Every third day, flies were transferred to new tubes containing fresh medium and deaths were scored. Survival rate graph was plotted with percentage of survival flies against days. Approximately 260 flies were tested per each genotype.

3. Results and discussion

3.1. Loss of *Drosophila* TDP-43 affects locomotive behaviors and life span

To suppress the *drosophila* TDP-43 gene (TBPH-CG10327) we generated chromosomal deletions from TBPH^{EY10530} (see Section 2). Two of these excised lines, TBPH^{Δ23} and TBPH^{Δ142} showed small 1.6 and 0.8 kb deletions, respectively, that partially removed

TBPH coding and regulatory regions (Fig. 1A). These deletions completely abolished endogenous protein expression and were therefore considered null alleles of TBPH (Fig. 1B). Homozygous TBPH^{Δ23} or TBPH^{Δ142} flies were viable after embryogenesis and more than 60% of them arrived to pupal stages, with the majority (~80%) undergoing metamorphosis. Nevertheless, a high percent of TBPH mutant animals were unable to eclose and remained trapped inside their pupal cages (Table 1). Homozygous flies that instead managed to get rid of the external cuticle were morphologically identical to wild type controls (Fig. 1C), however, they presented dramatic locomotive defects with spastic, uncoordinated, movements, incapacity to fly or walk normally and reduced life span (Fig. 1D and Supplementary Movies S1 and S2).

To determine the place of TBPH function, we explored its endogenous distribution and intracellular localization. TBPH protein was present at higher concentrations in head tissues compared with thorax or abdomen in adult flies (Fig. 1E). Similarly, our anti-TBPH antibody could detect the endogenous protein in

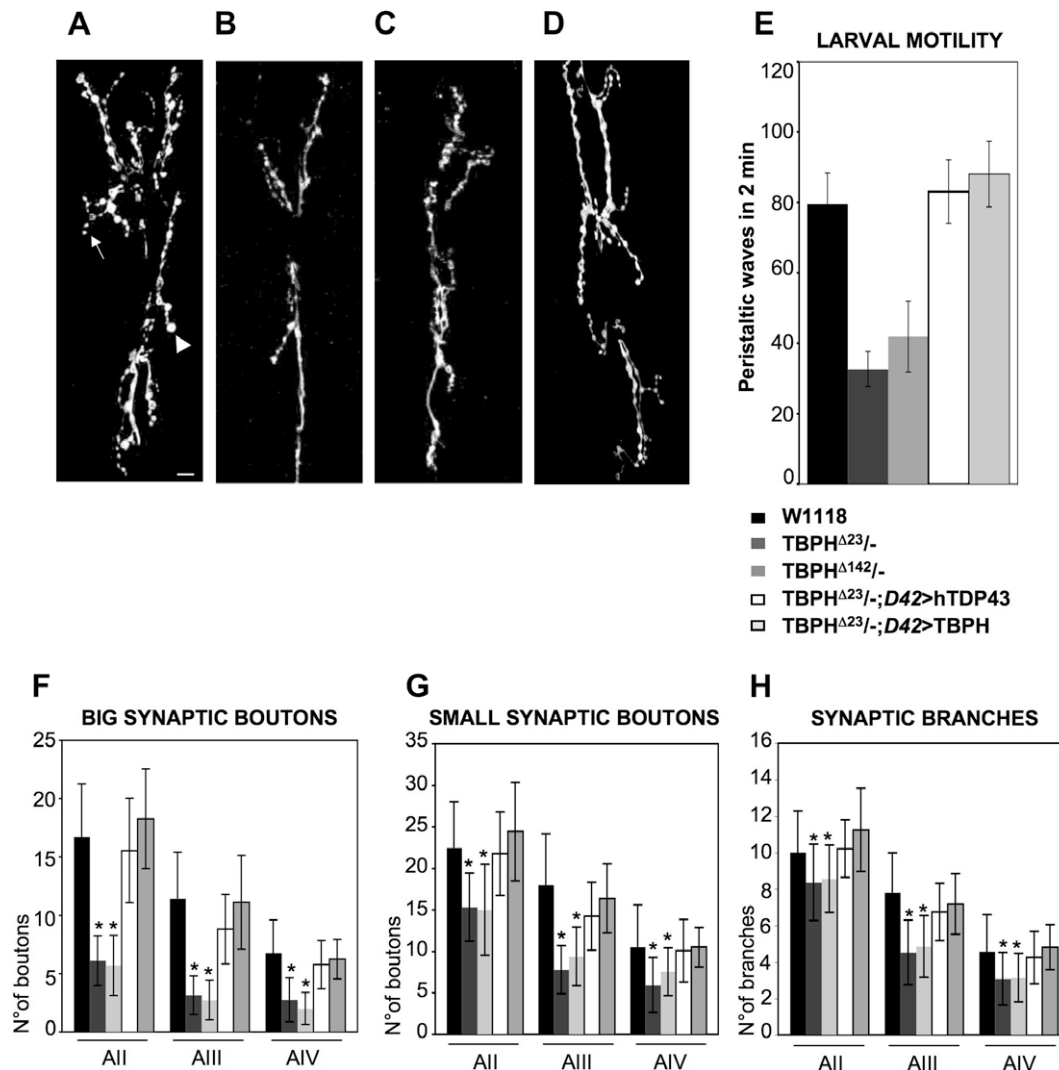


Fig. 4. Morphological defects at neuromuscular synapses in TBPH mutant flies. (A) Confocal images of motoneurons presynaptic terminals at muscles 6 and 7 (abdominal segment III) in wild type third instar larvae stained with anti-HRP antibodies, reveals the branching pattern and the presence of big (arrowhead) and small (arrow) synaptic boutons. (B) and (C) Similar staining and anatomical position for TBPH^{Δ23} and TBPH^{Δ142} homozygous larvae respectively, show reduced axonal branching pattern and number of synaptic boutons. (D) TBPH^{Δ23} minus third instar larvae rescued by expressing UAShTDP-43 in motoneurons with D42-GAL4 shows recovery of presynaptic complexity with increased formation of synaptic boutons and axonal terminal branching. Magnification 63×. (E) Number of peristaltic waves observed during 2 minutes in 120 h third instar larvae. *n* = 20 for each genotype, error bars indicate S.D.; *P* < 0.0001 calculated by ANOVA. (F) Quantification of big synaptic boutons present in consecutive abdominal segments (*n* = 20 animals). (G) Analysis of small synaptic boutons (*n* = 20 animals). (H) Quantification of presynaptic terminals branches in wild type, TBPH minus and hTDP-43/TBPH rescued third instar larva. *n* = 20 animals for each genotype, error bars indicate S.D.; *P* < 0.0001 calculated by ANOVA single factor. Scale: 10 μm.

neuronal cells during larval development and adulthood (Fig. 2). TBPH staining showed very well defined spherical structures that may correspond to the cell nucleus inside neuronal cell bodies present in optic lobes (Fig. 2i and ii) midbrain areas and ventral ganglia of larval (Fig. 2v and vi) and adult brains (Fig. 2ix and x). Identical immunostaining performed in TBPH minus tissues did not detected any particular signal confirming the specificity of the observations described above (Fig. 2iii, iv, vii, viii, xi and xii). These experiments demonstrate that TBPH is highly expressed in neuronal tissues from developmental stages to adulthood and suggest that its loss of function may induce locomotive deficits of neurological origin.

3.2. TDP-43 function in neurons regulates flies locomotion

To test this hypothesis, we decided to suppress TBPH expression by RNA interference (RNAi) exclusively in neural tissues [13]. Expression of TBPH RNAi using two different pan-neuronal drivers *elav-GAL4* and *1407-GAL4* [14], consistently reduced endogenous TBPH protein levels in *Drosophila* heads and induced locomotive defects in climbing assays (Fig. 3A and B). More convincingly, we observed that these phenotypes became enhanced if similar RNAi treatments were applied to TBPH heterozygous flies (see *elav > RNAi3v*; TBPH^{Δ23}/+ in Fig. 3C and Supplementary Movie S3), indicating that suppression of TBPH activity in post mitotic neurons was the presynaptic activity of sufficient to induce locomotive defects in vivo. To confirm these results we decided to rescue TBPH minus phenotypes by reintroducing the deleted gene. In addition, we decided to test whether the human protein (hTDP-43) was able to replace the endogenous protein in vivo. For these experiments we generated transgenic flies containing flag tagged TBPH and hTDP-43 cDNAs and targeted their expression in TBPH minus backgrounds using the GAL4/UAS system (Fig. 3D). Strikingly, we found that the expression of hTDP-43 by the neuronal post mitotic driver *elav-GAL4* managed to rescue the motility defects observed in TBPH homozygous flies to a situation similar to wild type (Fig. 3C, and Supplementary Movie S4). Moreover, we found that expression of hTDP-43 in a more restricted population of neurons that include motoneurons by *D42-GAL4* [15], was sufficient to rescue TBPH null phenotypes. In fact, these flies recovered their locomotive capacities (Fig. 3C, and Supplementary Movie S5) and incremented their life span (Fig. 1D) demonstrating that hTDP-43 function is evolutionary conserved and sufficient in a limited subpopulation of neurons to restore these traits. However, the partial recovery of the life span observed in these *D42-GAL4* expressing flies indicates that TDP-43 may also be required in other types of neurons or different tissues.

3.3. TDP-43 regulates the formation of motoneurons presynaptic terminals at NMJ

To gain insight into the mechanisms behind these motility defects, we decided to analyze the morphology of motoneurons presynaptic terminals at neuromuscular junctions (NMJ) in TBPH minus flies. To label these structures we used anti horseradish peroxidase antibodies (HRP) that label neuronal membranes [16] and quantified the synaptic pattern of motoneuron axons that innervate muscles 6 and 7 in three different larval abdominal segments (AII, AIII, AIV). These structures present two types of synaptic boutons that can be easily distinguish by their size in 1b (big) and 1s (small) (Fig. 4A) [17]. We observed that in TBPH minus larvae, the complexity of the presynaptic terminals became dramatically affected as reflected by the reduced number of axonal branches and synaptic boutons present inside the muscles (Fig. 4B and C, F–H). In addition, we found that these anatomical defects together with the functional problems in larval motility could be rescued by

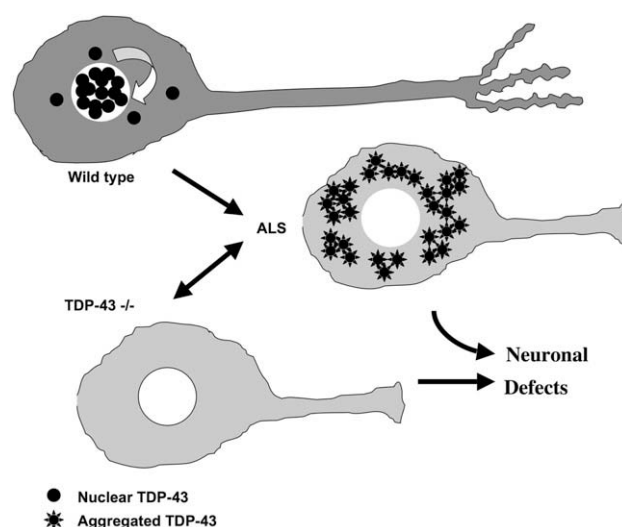


Fig. 5. Possible mechanism of TDP-43 mediated neurological defects in ALS. TDP-43 is a nuclear protein that in wild type situation shuttles to the cytoplasm. In ALS brains instead, it becomes abnormally aggregated in the cytoplasm and depleted from the cell nucleus suggesting that these modifications may be related to the neurological problems observed in these patients. We found that depletion of *Drosophila* TDP-43 was sufficient to generate atrophic presynaptic terminals and to induce locomotive defects in vivo. Since the human TDP-43 protein was able to assume the role of the endogenous gene in flies, we hypothesize that nuclear loss of TDP-43 function may be related to the locomotion problems observed in the disease. This view provides a new interpretation of the pathological mechanisms in ALS and predicts similar defects at neuromuscular junctions in affected patients.

the expression of hTDP-43 under *D42-Gal4* (Fig. 4D and E) indicating that the morphological modifications observed at the synaptic terminals are the basis of these behavioral problems and TBPH/hTDP-43 function is required to form and maintain these structures.

Thus, our results demonstrate that the activity of TDP-43 in neurons is necessary to regulate locomotive behaviors in vivo and indicates that the neurological problems observed in ALS patients may not be restricted to the formation of insoluble protein fragments of aggregated TDP-43 within the cytosol but also to the loss of TDP-43 function in the nucleus (see model in Fig. 5).

In view that TDP-43 was characterized as RNA binding protein of the heterogeneous nuclear ribonucleoproteins (hnRNP) family with splicing inhibitory capacity [18], it may well be that TDP-43 induce motoneurons defects by affecting spliceosomes activity and/or mRNA transport and localization. Similarly, multiple defects in mRNA processing associated with locomotive deficiencies were recently described for the survival motor neuron protein (SMN) [19], giving a major support to the idea that defects in the mRNA metabolism may play a significant role in motoneuron degeneration. In conclusion our data demonstrate an evolutionary conserved function of TDP-43 in regulating synaptic terminals and locomotive behaviors and provide a new model to understand the mechanisms that lead to TDP-43 mediated neurological diseases in vivo.

Acknowledgments

To Emanuele Buratti and members of the Baralle's lab for useful discussions. To Serena Zacchigna and Chiara Appocher for advice and help. This work was supported by the Ministero dell'Università e della Ricerca (Grant No. RBLA03AF28-003), Telethon Onlus Foundation (Grant No. GGP06147) and by a European community grant (EURASNET-LSHG-CT-2005-518238).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at [doi:10.1016/j.febslet.2009.04.019](https://doi.org/10.1016/j.febslet.2009.04.019).

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